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Free radical scavenging and antioxidant activity of D-pinitol against 7, 12 dimethylbenz(a) anthracene induced breast cancer in sprague dawley rats

Thamaraiselvan Rengarajan[1,2], Peramaiyan Rajendran[2], Natarajan Nandakumar[1,3], Maruthaiveeran Periyasamy Balasubramanian[1*], Ikuo Nishigaki[2]

[1]*Department of Pharmacology and Environmental Toxicology, Dr. ALM PG IBMS, University of Madras, Chennai-600113, Tamilnadu, India*

[2]*NPO-International Laboratory of Biochemistry, 1-166, Uchide, Nakagawa-ku, Nagoya 454-0926, Japan*

[3]*Department of Microbiology, Immunology and Genetics, Ben Gurion University of the Negev, Beer Sheva, 84105, P.O.B.653, Israel*

PEER REVIEW

Peer reviewer

Dr. Mahesh Ramalingam, Oriental Medicine R&D Center, Dongguk University, Gyeongju, 780-714, Republic of Korea.
E-mail: melanimahesh@gmail.com

Comments

This is a good study in which the authors evaluated the free radical-scavenging property exhibited by D-Pinitol may be one of the mechanisms by which this drug is useful as a foodstuff and as well as usefulness against free radical mediated diseases.

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ABSTRACT

Objective: To investigate whether D-pinitol efficiently scavenge free radicals using various *in vitro* models [1, 1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide, superoxide anion and total antioxidant activity] and *in vivo* models.

Methods: Female Sprague-Dawley rats (150-160 g) were divided into four groups and each group consisting of six animals. Group I and group IV were vector and drug control. The group II and group III animals were treated with 7, 12-dimethylbenz(a)anthracene (DMBA) 20 mg/kg body weight to induce mammary carcinoma. Rats received cancer bearing Group III animals were treated with D-pinitol at the concentration of 200 mg/kg bodyweight for 45 d orally. Five different concentrations of D-pinitol (100 to 500 μg/mL) were used in *in vitro* studies.

Results: The results revealed that D-pinitol efficiently scavenges DPPH radicals and the IC_{50} was found to be 290 μg/mL and it also exhibited a dose dependent antioxidant activity at different concentrations. In addition, the superoxide and nitricoxide radicals were also significantly inhibited by D-pinitol at the concentrations of 360 and 390 μg/mL respectively. On the other hand, D-pinitol has significantly increased antioxidant enzymes during DMBA induced mammary carcinoma.

Conclusions: It can be concluded from the investigation that D-pinitol has an excellent antioxidant activity which could be due to the scavenging capacities on the stable DPPH radicals, superoxide, nitric oxide and DMBA induced free radicals thereby it exhibits remarkable total antioxidant activity.

KEYWORDS

D-pinitol, Free radicals, DPPH, Federal trade commission, Nitricoxide, Superoxide, DMBA

1. Introduction

In living organisms various reactive oxygen species (ROS) can be formed by different ways. In normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides[1]. Oxidative damages play a significantly pathological role in human diseases. It has been shown that many degenerative diseases such

*Corresponding author: Dr. M. P. Balasubramanian, Department of Pharmacology and Environmental Toxicology, Dr. ALM PG IBMS, University of Madras, Chennai-600113, Tamilnadu, India.
Tel: +91-44-24547143
Fax: +91-44-24540709

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as cancer, emphysema, cirrhosis, arteriosclerosis and arthritis have been correlated well with oxidative damage[2]. Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers[3]. However, antioxidant supplement may be used to the help the human body to reduce the oxidative damage. Most living species have an efficient defense system to protect themselves against the oxidative stress induced by ROS[4]. Therefore, the development and utilization of more effective antioxidants of natural origin are desired. In this context, natural compounds especially from the dietary origin have been shown to have efficient therapeutic activities in various experimental studies. D-pinitol is one of the natural compound and major constituent isolated from the soyabean[5], *Bougainvillea spectabilis* and *Pinus lambertian*[6,7]. The present study was designed with an objective to investigate the scavenging capacity of D-pinitol on the stable DPPH free radicals, superoxide anion radicals, nitric oxides and total antioxidant activity by *in vitro* analysis and to evaluate the *in vivo* antioxidant activity of D-pinitol during 7,12 Dimethylbenz(a) anthracene (DMBA)induced mammary carcinoma in Sprague Dawley rats.

2. Materials and methods

2.1. Chemicals

D-pinitol, DMBA, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and Linoleic acid were purchased from Sigma Chemicals Company, USA. All the other chemicals used in the study were of high purity and analytical grade marketed by Sisco Research Laboratories Pvt, Ltd, Mumbai, India.

2.2. In vitro antioxidant assay

2.2.1. DPPH radical-scavenging activity

The antioxidant activity of D-pinitol was performed based on the scavenging activity of the stable DPPH free radical according to the method of Hatano *et al*. (1989)[8]. Vitamin-C was used as standard. The percentage of free radical inhibition activity was calculated as IC_{50}. The percentage radical scavenging activity was calculated from the following formula:

% Scavenging DPPH=$[(A_0-A_1)/A_0]\times100$.

The results were expressed as % inhibited/μg of D-pinitol.

2.2.2. Superoxide scavenging activity

Measurement of superoxide anion scavenging activity of D-pinitol was assayed based on the method described by Liu *et al*. with slight modifications according to Gulcin *et al*[9,10]. Vitamin C was used as standard. The percentage inhibition of superoxide anion generation was calculated using the following formula:

% Inhibition=$[(A_0-A_1)/A_0]\times100$

The results were expressed as % inhibited/μg of D-pinitol.

2.2.3. Nitric oxide scavenging activity

Nitric oxide scavenging activity of D-pinitol was assayed based on the method described by Marcocci *et al*[11].Vitamin C was used as standard. The percentage inhibition of nitric oxide radical generation was calculated using the following formula:

% Inhibition=$[(A_0-A_1)/A_0]\times100$

The results were expressed as % inhibited/μg of D-pinitol.

2.2.4. Total antioxidant activity

The total antioxidant activity of D-pinitol was determined according to Ferric thiocyanate method of Mitsuda *et al*. and Osawa and Namiki with sight modification according to Kikuzaki and Nakatani[12-14]. Vitamin E was used as standard. The results were expressed as % inhibited/μg of D-pinitol.

2.3. In vivo analysis

2.3.1. Animals

Female Sprague-Dawley rats at the age group of 45-48 d were procured from the Central Animal House Facility, Dr.ALM PGIBMS, University of Madras, Taramani. The animals were housed in well ventilated large spacious polypropylene cages and had 12 h light and dark cycle throughout the experimental period. The animals received a balanced diet of commercially available pellet rat feed and water *ad libitum*. The Guidelines for Breeding and Experiments on Animals, 1998 defined by the Ministry of Social Justice and Empowerment of India were followed and the protocol was approved by the Institutional Animal Ethics Committee (IAEC No. 01/05/2011).

2.3.2 Tumor induction

DMBA was used as a carcinogen for the present investigation. Mammary tumor was induced by a single dose of 20 mg of DMBA dissolved in corn oil (1 mL) given through an oral gavage[15].

2.3.3 Experimental design

The rats were divided into four groups and each group consisting six animals. Group I rats received single dose of 1 mL of emulsion of corn oil given orally throughout the experimental period, served as vehicle treated control. Rats in Groups II and III were induced mammary carcinogenesis by providing single dose of 20 mg/kg body weight of DMBA in 1 mL emulsion of corn oil intragastrically. Group II rats received no other treatment. After 60 d the cancer bearing Group III rats received D-pinitol at the concentration of 200 mg/kg bodyweight for 45 d orally. Group IV rats were treated with D-pinitol alone at the concentration of 200 mg/kg body weight for 45 d orally.

2.3.4. Collection of Samples

At the end of the experimental period all the rats were sacrificed by cervical dislocation. The erythrocyte membrane was prepared by the method of Dodge *et al*. with a change in buffer, according to Quist[16,17]. Blood was collected with ethylene diamine tetraacetic acid was centrifuged at 2000 r/min for 20 min at 4 °C. The packed cells were washed with isotonic saline to remove buffy coat. An aliquot of 1.0 mL washed cells was lysed using 9.0 mL of isotonic Tris-HCl (0.3

mol/L, pH 7.4) buffer. An aliquot of 1.0 mL washed cells lysed using 9.0 mL of hypotonic Tris-HCl buffer (0.015 mol/L, pH 7.2). The lysed cells were centrifuged for 30 min at 15000 r/min. The pellet was repeatedly washed with hypotonic Tris-HCL buffer until a clear pale pink or colorless supernatant was obtained. The resulting erythrocyte membrane pellet was suspended in 0.01 mol/L Tris-HCL buffer (pH 7.4). Aliquots from these preparations were used for the biochemical estimations.

2.3.5. Estimation of enzymic antioxidants

The activity of superoxide dismutase (SOD) was determined by the method of Marklund and Marklund[18]. The absorbance was read at 420 nm using a spectrophotometer as the degree of inhibition of autoxidation of pyrogallol in an alkaline pH. The catalase (CAT) activity measured by dichromate in acetic acid was reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2) with the formation of perchloric acid as unstable intermediate and chromic acetate thus formed which was measured spectrophotometrically at 570 nm by the method of Sinha[19]. The results were expressed in terms of μmol of H_2O_2 liberated/min/mg Hb. The activity of glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al*[20]. Oxidation of glutathione by the enzyme was measured spectrophotometrically at 420 nm. The activity of GPx was expressed as μmol glutathione oxidized/min/mg Hb.

2.3.6. Estimation of non- enzymic antioxidants

The level of reduced glutathione was estimated by the method of Moron *et al.* (1979)[21]. The sample (1.0 mL) was precipitated with 1.0 mL of TCA and centrifuged at 1200×*g* for 20 min. To 0.5 mL of supernatant 2.0 mL of 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) was added and the color developed was read immediately at 412 nm using a spectrophotometer. The amount of glutathione in tissue was expressed as μmoles/mg/dl. Vitamin E (α-tocopherol) levels were estimated by the method of Desai where the ferric ions are reduced to ferrous ions in the presence of tocopherol and bathophenanthroline to form a pink coloured substance[22], which was read at 530 nm using a spectrophotometer. The level of vitamin E was expressed as mg/dL.

The levels of vitamin C (ascorbic acid) were assayed by the method of Omaye *et al*[23]. In this reaction 1.0 mL of sample was added with 1.0 mL of 10% TCA to mix thoroughly and then centrifuged at 3500 r/min for 20 min. The supernatant (2.0 mL) was mixed with 0.2 mL of 2,4-dinitrophenyl hydrazine-thiourea-copper sulphate (DTC) and 65% H_2SO_4. The absorbance was measured at 520 nm. The level of vitamin C was expressed as mg/dL.

2.4. Statistical analysis

Experimental results were mean±SD of three parallel measurements. Analysis of variance was performed by ANOVA procedures (SPSS 9.0 for Windows). Significant differences between means were determined by Duncan's Multiple Range tests. $P<0.05$ were regarded as significant and $P<0.01$ very significant.

3. Results

3.1. DPPH radical scavenging activity of D-pinitol

The antioxidant activity of D-pinitol is performed by using the DPPH method and the results are presented in Figure 1. From the result it was found that D-pinitol showed excellent antioxidant activity in a dose dependent manner at various concentrations such as 100, 200, 300, 400 and 500 μg/mL. Interestingly the D-pinitol exhibits nearly 50% excellent radical scavenging activity at the concentration of 290 μg/mL, when compared with the standard vitamin C .

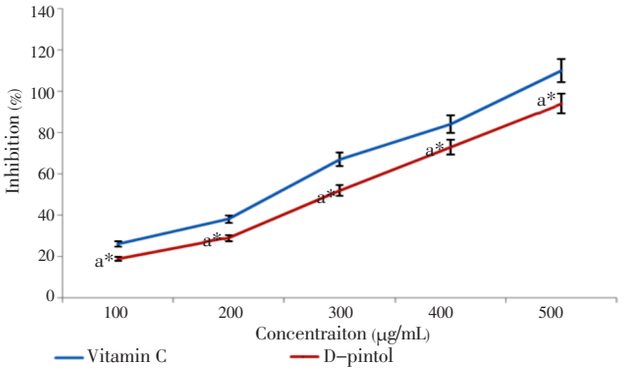

Figure1. DPPH radical scavenging activity of D-pinitol.
Values are mean±SD of triplicate determinations. *$P<0.05$ when compared against control.

3.2. Superoxide scavenging activity of D-pinitol

Figure 2 depicts the inhibitory effect of D-pinitol on the superoxide anion formation. The scavenging effects of D-pinitol on the superoxide anion formation were examined at five different concentrations (100, 200, 300, 400 and 500 μg/mL). D-pinitol showed remarkable inhibition of superoxide anion formation in a dose dependent manner and maximum inhibitions of superoxide were observed at the concentration of 360 μg/mL and were comparable with vitamin C.

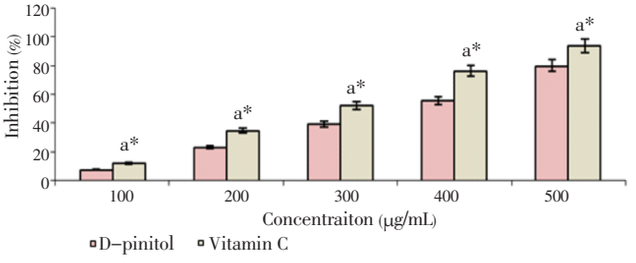

Figure 2. Super oxide scavenging activity of D-pinitol.
Values are mean±SD of triplicate determinations. *$P<0.05$ when compared against control.

3.3. Nitricoxide scavenging activity of D-pinitol

The results of the nitricoxide scavenging activity of D-pinitol are presented in Figure 3. It was found that the nitric oxides were significantly scavenged by the D-pinitol at various concentrations such as 100, 200, 300, 400 and 500 μg/mL with the remarkable inhibition of nitric oxide generation observed at the concentration of 390 μg/mL when compared with vitamin C.

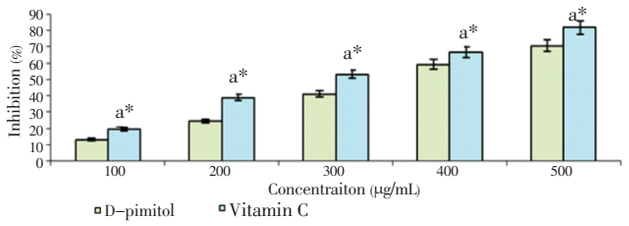

Figure 3. Nitric oxide scavenging activity of D-pinitol.

Values are mean±SD of Triplicate determinations. *P<0.05 when compared against control.

3.4. Total antioxidant activity of D-pinitol by FTC method

The total antioxidant activity of D-pinitol by Federal Trade Commission (FTC) method is presented in Table 1. The results revealed that the D-pinitol markedly inhibited the oxidation of linoleic acid for the period of 5 d with nearly 50% of inhibition when compared to the standard vitamin E and the results were found to be statistically significant and was comparable to control.

Table 1

Total antioxidant activity of D-Pinitol by FTC method.

Days	Control	Inhibition of vitamin C (%)	Inhibition of D-pinitol (%)
Day 1	0.04±0.001	0.082±0.020$^{\#}$	0.610±0.004$^{\#}$
Day 2	0.14±0.002	0.130±0.007$^{\#}$	0.093±0.007$^{\#}$
Day 3	0.25±0.001	0.195±0.005$^{\#}$	0.192±0.002$^{\#}$
Day 4	1.67±0.010	0.270±0.006$^{\#}$	0.245±0.003$^{\#}$
Day 5	5.31±0.020	0.530±0.002$^{\#}$	0.496±0.004$^{\#}$

Values are mean±SD of six parallel measurements. $^{\#}$: P<0.05 when compared against control.

3.5. Antioxidants

Table 2 shows the activities of enzymatic antioxidants (SOD, CAT, GPx) and non-enzymatic antioxidants [reduced glutathione (GSH), vitamin-C, vitamin-E] level in erythrocytes of control and experimental animals in each group respectively. The activities of SOD, CAT, GPx and GSH, vitamin-C, vitamin-E level were significantly decreased in erythrocytes of cancer bearing animals (Group II) as compared to control animals. Oral administration of D-pinitol reverted the activities of enzymatic and non-enzymatic antioxidants level to near normal range. However no significant changes were observed in D-pinitol alone treated animals.

Table 2

Effect of D-pinitol on enzymic and non-enzymic antioxidants in the erythrocytes of control and experimental animals.

Parameters	Group I (Control)	Group II (DMBA)	Group III (DMBA+D-Pinitol)	Group IV (D-Pinitol)
SOD	12.84±0.44	7.67±0.35^{a*}	10.12±0.98^{a*b*}	13.19±0.07^{aNS}
CAT	46.64±2.41	28.13±1.60^{a*}	37.97±5.39^{a*b*}	48.51±2.53^{aNS}
GPx	10.96±0.65	4.12±0.78^{a*}	7.27±0.16$^{a*b\#}$	9.79±0.34^{aNS}
GSH	9.19±1.42	5.67±0.43^{a*}	7.19±0.12^{a*b*}	8.09±0.69^{aNS}
Vitamin C	2.64±0.31	1.02±0.91^{a*}	1.84±0.11$^{a@b*}$	2.19±0.25^{aNS}
Vitamin E	1.78±0.02	0.76±0.73^{a*}	1.26±0.82^{a*b*}	1.47±0.41^{aNS}

Unit are expressed as: SOD=units/mg protein; CAT=μmol of H_2O_2 consumed/mg protein/min; GPx=μg of GSH utilized/mg protein/min; GSH=μg/mg protein/min; Vitamin E and Vitamin C=mg/dL. Each value represents mean±SD of six animals. a: Group II, III and IV compared with Group I. b: Group III compared with Group II. Statistical significance: $^{@}P$<0.001; $^{\#}P$<0.01; $^{*}P$<0.05; NS: No significant.

4. Discussion

ROS are formed continuously in cells due to both oxidative biochemical reactions and external factors. However, they become harmful when they are produced in excess under certain abnormal conditions such as inflammation, ischemia and in the presence of catalytic ions of iron. Under these stressed conditions, the endogenous antioxidants may be unable to counteract ROS formation[24]. Additionally, Forman *et al.* has also suggested that ROS formed may cause cellular damage by peroxidation of membrane lipids[25], sulfhydryl enzyme inactivation, protein cross-linking and DNA breakdown. Dietary antioxidants are believed to play an important role in the prevention and treatment of a variety of diseases associated with oxidative stress.

DPPH is a stable free radical, which has been widely accepted tool for estimating free radical scavenging activities[26]. This method is based on the reduction of DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form of reaction. DPPH accepts an electron from hydrogen radical to become a stable diamagnetic molecule. In this context, Roopa *et al.* have reported that the decreases in radical absorbance by DPPH caused by antioxidants result in the scavenging of the radical by hydrogen donations, which is visually noticeable as a discoloration from violet to yellow[27]. Interestingly, we have observed a noticeable discoloration from violet to yellow up on D-pinitol exposure to DPPH radicals. This clearly indicated the antioxidant activity of the D-pinitol. The nitric oxide radicals play an important role in inducing inflammatory response and their toxicity multiplies only when they react with superoxide anion radicals to form peroxynitrite, which damages biomolecules such as, proteins, lipids and nucleic acids[28]. Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite. Incubation with D-pinitol inhibits nitrite formation by competing with oxygen to react with nitricoxide directly. In the present study, the D-pinitol was active and it might act as very potent and novel therapeutic agent for scavenging nitric oxide. Superoxide is a well-known free radical of all oxygen-derived species[29]. It is the first intermediate in the sequential univalent reduction of oxygen that leads to form hydrogen peroxide. Superoxide radical is unique in that it can lead to form many other reactive species, including hydroxyl free radical, hydrogen peroxide and perhydroxyl radicals[30]. It induces oxidative damage in lipids, proteins and DNA[31].The most important source of superoxide is oxidative enzymes, among which xanthine oxidase and NADPH/NADH oxidae are the most effective sources. On the other hand it also produces in the extra cellular fluid by macrophages during phagocytosis in the inflammatory phase of diseased conditions[32]. In the present study, incubation with D-pinitol results in a decrease of absorbance, which indicates the consumption of superoxide anion in the reaction mixture. The decreases in absorbance level of the present investigation may be due to the antioxidant activity of D-pinitol. In addition, the total antioxidant activity of D-pinitol was measured by FTC method. The FTC method was used to measure the amount of peroxide at the beginning of lipid peroxidation,

in which peroxide reacts with ferrous chloride and forms ferric ions[33]. In the present investigation D-pinitol showed low absorbance values, which indicated a high level of antioxidant activity. The end point of FTC method indicated the presence of antioxidant activity of D-pinitol which inevitably proved that D-pinitol had excellent antioxidant activity *in vitro*. The erythrocytes are more prone to oxidative damage due to high content of iron and polyunsaturated fatty acids and their role as O_2 transporters. Furthermore, the erythrocytes have been suggested to act as sinks for H_2O_2 and O_2-produced in the plasma[34]. However, the erythrocytes and plasma are richly endowed with efficient antioxidant defense mechanisms. The antioxidant enzymes, SOD widely distributed in all cells are present in high amounts in erythrocytes[35]. This enzyme protects the red cells against $O_2^{\bullet -}$ and H_2O_2 mediated lipid peroxidation[34]. SOD is said to act as the first line of defense against superoxide radical generated as a by-product of oxidative phosphorylation[36]. In the present study there was multi-fold ability of the free radicals resulting in the increase of the levels of hydroxyl free radicals in DMBA induced Group II animals that was neutralized to near normal in D-pinitol treated Group III animals, thus indicating prevention of increased ROS production by the D-pinitol. CAT primarily decomposes hydrogen peroxide to water at a much faster rate, sharing this function with GPx. GPx may play an important role in the removal of lipid hydroperoxides. The balances between these enzymes are important for the efficient removal of oxygen radicals from tissues[37]. The significant elevation in the level of both CAT and GPx in D-pinitol treated group III animals were observed. This which indicates D-pinitol plays an important role in antioxidant defense system. The second line of defense consists of the non-enzymic scavengers such as glutathione, ascorbic acid and α-tocopherol, which scavenge residual free radicals escaping from decomposition by the antioxidant enzymes. Moreover, enzymic antioxidants are inactivated by the excessive levels of free radicals and hence the presences of non-enzymic antioxidants are presumably essential for the removal of these radicals[38]. GSH which is an important non-protein thiol in conjugation with GPx and GST plays a significant role in protecting cells against cytotoxic and carcinogenic chemicals by scavenging reactive oxygen species[38,39]. Higher levels of GSH and over expression of GSH-dependent enzymes have been reported in DMBA induced tumors[40]. In this context, Nishikawa is of the opinion that tumor cells can generate and release ROS into circulation and the peroxides released from tumor cells can subsequently oxidize GSH[41]. This may be one of the reasons for the decreased concentration of GSH was observed in Group II cancer bearing animals. In D-pinitol treated rats, a significantly increased level of GSH was observed when compared to DMBA induced animals, which may be due to the antioxidant property of D-pinitol. Ascorbic acid is an essential antioxidant that disappears faster than other antioxidants when plasma is exposed to ROS[42]. Vitamin E is the major lipid soluble antioxidant present in plasma and erythrocyte membranes[43]. Generally, the protective effect of vitamin E is a result of the inhibition of free radical formation and activation of endonucleases. This shows that D-pinitol maintains the levels of antioxidant vitamins by maintaining GSH homeostasis, thereby protecting the cells from further oxidative stress. This is an indication of the potent antioxidant activity possessed by D-pinitol. In addition Sivakumar *et al*. reported that D-pinitol effectively scavenge free radicals in diabetic treated animals[44].

It can be concluded from the present investigation that, the natural compound D-pinitol excellently exhibited different levels of antioxidant activity in all the *in vitro* and *in vivo* models employed and also the results revealed that the D-pinitol had significant antioxidant activity through quenching various free radicals. The free radical-scavenging property exhibited by D-pinitol may be one of the mechanisms by which this drug is useful as a foodstuff and as well as usefulness against free radical mediated diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from naturals sources. Many of them are based on their use in traditional medicine. In the present study, the medicinal and nutritional importance of D-pinitol has been investigated and found to be quite interesting.

Research frontiers

The present study was designed with an objective to investigate the scavenging capacity of D-pinitol on the stable DPPH free radicals, superoxide anion radicals, nitric oxides and total antioxidant activity by *in vitro* analysis and to evaluate the *in vivo* antioxidant activity of D-pinitol during DMBA induced mammary carcinoma in Sprague Dawley rats.

Related reports

The results of the present study are in agreement with Sivakumar *et al.* (2010) who found that antioxidant property of D-pinitol in diabetic induced rats.

Innovations & breakthroughs

Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also acting as oxygen scavengers. However, antioxidant supplement may be used to help human body to reduce the oxidative damage. Most living species have an efficient defense system to protect themselves against the oxidative stress induced by ROS. Therefore, the development and utilization of more effective antioxidants of natural origin are desired.

Applications

Oxidative damages play a significantly pathological role in human diseases. It has been shown that many degenerative diseases such as cancer, emphysema, cirrhosis, arteriosclerosis and arthritis have been correlated well with oxidative damage. Furthermore, excessive generation of ROS induced by various stimuli leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity and cancer.

Peer review

This is a good study in which the authors evaluated the free radical-scavenging property exhibited by D-pinitol may be one of the mechanisms by which this drug is useful as a foodstuff and as well as usefulness against free radical mediated diseases.

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